

UNIVERSIDADE DE LISBOA
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B cell gene expression in rheumatoid arthritis:
Effect of immunosuppressive treatment

Ana Rita Fernandes Vieira

Dissertação

Mestrado em Biologia Molecular e Genética

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Ana Rita Fernandes Vieira

Dissertação orientada por:

Doutora Rita Alexandra Pedra Aguiar de Moura
Instituto de Medicina Molecular (IMM)

Professora Doutora Maria Margarida BlasquesTelhada
Faculdade de Ciências da Universidade de Lisboa

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ACKNOWLEDGEMENTS

While writing this thesis I benefited greatly from the discussion I had with a number of people, some of whom also commented parts of it. First, I would like to thank to Professor João Eurico Fonseca and Professor Helena Canhão for receiving me in the Rheumatology Research Unit and for all the ideas brought to the work throughout this year. Also, thanks to Professor Margarida Telhada for having agreed to be my internal supervisor, even in the last minute.

I owe a special debt of gratitude to Rita Moura for accepting me as her student and for the stimulating and fruitful conversations we had, not only on the subject but also in other areas, as well as the encouragement and support she gave me in the preparation of the thesis. Despite being a morning person I learned that the nights in the lab can be less crowded, and, therefore, more quiet than the afternoons. To Claudia because I know now how important it is to have someone in the lab to share our doubts, to help each other, to discuss at length the results while we waited to acquire all events at FACS... as real lab partners do!

A research unit is nothing without its students, whether they are PhD, MSc, graduates or even other people that manage and contribute to the development of it. Our paths were crossed and because of that, I share my 'thank you' to all those who had to, somehow, deal with my uncertainties. I might be forgetting someone, but here it is (and the order is arbitrary): Inês, Bruno, Ana and my other mates for creating a wonderful work environment, Mónica, Rita R., Ana D. and Joana S. – thanks y'all!

Because *we need those who care about us to provide a mirror for our actions as we step out into the brave new world*¹, I have to thank my family for the support especially my parents who have been giving me opportunities to pursue my dreams and were always here for every milestone I stepped into.

Finally I would like to thank to my long-time friends that, in their ways, tried to understand my enthusiasm for investigation. Sara thanks for all those years of friendship and for the person who I could always count to get something off my chest; you became almost like the sister I wish to have had.

In this last two years new friendships were made and now the team "Potência máxima" is constantly nagging me (in a good way) about the writing and giving me helpful advices. After all, you already went through the same process and understand better than anyone what this type of endeavour means. For that and much more I will never forget you...

I hope that the project contained in this thesis seems of any merit.

*It might be true that there are six billion people in the world, and counting.
Nevertheless, what you do makes a difference.* Waking life (2001)

¹ Intern: A Doctor's Initiation, Sandeep Jauhar

RESUMO

A artrite reumatoide (AR) é uma doença autoimune sistêmica que se caracteriza pela inflamação crônica das pequenas articulações, particularmente das mãos, bem como articulações maiores, que incluem os joelhos e tornozelos. A prevalência da doença é de cerca de 1% na população mundial adulta e é mais frequente nas mulheres do que nos homens (numa proporção de 3:1). Se não for tratada, a AR origina destruição articular, com erosão óssea e da cartilagem.

O diagnóstico da AR é feito de acordo com critérios definidos pelo American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR), baseados numa avaliação radiológica e atribuição de pontuação relativa ao número de articulações afetadas; serologia de autoanticorpos IgM específicos para o fragmento Fc de imunoglobulinas IgG (fator reumatoide, RF) ou para péptidos cíclicos citrulinados modificados pós-traducionalmente por incorporação de citrulina por ação das peptidil arginina deaminases (anticorpos anti-proteínas citrulinadas, ACPA ou anti-CCP); quantificação de fatores de inflamação (velocidade de sedimentação eritrocitária e proteína-C reativa); e duração da doença.

A etiologia da doença é desconhecida, mas sabe-se que a influência do genótipo e do ambiente parecem ser fatores essenciais para o início do processo inflamatório. Em termos de predisposição genética, a AR está associada a mutações no gene *HLA-DRB1* que pertence ao complexo maior de histocompatibilidade (MHC) classe II, tendo sido identificada uma curta sequência de aminoácidos comum às proteínas codificadas por todos os alelos de risco - o *shared epitope*. A AR está também associada a outros genes como o *PTPN22* e *CTLA-4*, entre outros. Em relação aos fatores ambientais, a maior associação descrita está relacionada com o tabagismo, sendo que o risco parece ser maior em indivíduos portadores do *shared epitope*. O efeito da exposição a alguns agentes infecciosos é controverso, mas alguns estudos indicam que poderá levar à formação de complexos imunes, implicados na AR. Também algumas alterações hormonais na mulher, como a gravidez, amamentação e contraceção oral, podem influenciar o desenvolvimento da doença.

No processo inflamatório da AR ocorre uma resposta celular e humoral que contribui para o desenvolvimento da sinovite, ou seja, a inflamação da membrana sinovial, que é a membrana que reveste a articulação. A hiperplasia da sinóvia resulta de uma migração e excessiva infiltração de linfócitos T e B, plasmócitos, células dendríticas, macrófagos, neutrófilos e mastócitos, assim como da ativação dos FLS – *fibroblast-like synoviocytes* –

que produzem citocinas pro-inflamatórias, provocando a ativação de outras células. A ativação de macrófagos através dos recetores Fc gama por complexos imunes que se formam a partir de autoanticorpos (RF e ACPA), produzidos por células B autoreactivas, juntamente com estímulos derivados de células T activadas, conduz à libertação de citocinas como a IL-1, IL-6, IL-15, INF- γ e TNF. Algumas destas citocinas ativam células sinoviais residentes a produzir enzimas proteolíticas, como a colagenase e metaloproteinases da matriz que medeiam a destruição da cartilagem. A destruição óssea também ocorre devido ao aumento da atividade dos osteoclastos, ativados através das ligações RANK/ RANK-L.

Nos últimos anos, os linfócitos B têm sido alvo de investigação, particularmente após a eficácia clínica observada com a terapia de depleção de células B com rituximab (anticorpo monoclonal anti-CD20). De facto, os linfócitos B podem contribuir de diversas formas para o desenvolvimento da AR, tais como: 1) produção de autoanticorpos como o RF e os ACPA, que formam complexos imunes que se depositam nas articulações e causam inflamação; 2) apresentação de antígenos e ativação de linfócitos T; 3) secreção de citocinas; 4) ativação de osteoclastos que levam à erosão óssea; 5) imunoregulação por células B reguladoras, que produzem citocinas anti-inflamatórias (IL-10 e TGF- β 1) que inibem células T activadas e restauram o balanço T_H1/T_H2 .

Apesar da AR não ter cura, é fundamental estabelecer um diagnóstico precoce para que se possa proceder ao tratamento e reduzir os sintomas no doente e a progressão da doença o mais rapidamente possível. O tratamento da AR faz-se com anti-inflamatórios não esteroides (AINEs), sendo que estes apenas diminuem a atividade inflamatória; corticoesteróides; DMARDs - agentes antirreumáticos modificadores da doença – sintéticos, onde se encontram o metotrexato (MTX), sulfassalazina (SSZ), hidroxicloroquina (HCQ) e a leflunomida, ou biológicos, que são geralmente anticorpos monoclonais que bloqueiam determinadas citocinas ou recetores celulares. Entre os DMARDs biológicos aprovados para tratamento da AR estão, por exemplo, os anti-TNF (etanercept, infliximab, golimumab, adalimumab e certolizumab), o tocilizumab – TCZ (anti-receptor da IL-6) e o rituximab (anti-CD20). Os DMARDs são conhecidos por melhorarem a capacidade funcional global, reduzirem os danos radiológicos e diminuírem os valores clínicos e laboratoriais associados à inflamação. Nem sempre os doentes respondem favoravelmente à terapêutica, podendo apresentar efeitos secundários graves devido ao efeito imunossupressor e/ ou mantendo níveis de atividade de doença elevados, o que obriga muitas vezes a alterações no tratamento, procedendo-se a mudança entre anti-TNFs, ou mesmo para outros biológicos, como o TCZ e o rituximab.

O principal objetivo deste trabalho consistiu na análise de um grupo de genes relacionados com a ativação e sobrevivência dos linfócitos B (BAFF-R, TACI, BCMA), mudança de classe e hipermutação somática (AID), diferenciação de plasmócitos (BLIMP-1), quimiotaxia (CXCR5), apoptose (BCL- 2), inibição (CD32), inflamação (B2M) e ativação de células B através de *Toll-like receptors* (TLR7, TLR9, TLR10) em doentes não tratados com AR inicial (ERA) (≤ 1 ano de duração da doença) e na comparação da expressão dos mesmos genes com doentes com AR estabelecida após tratamento com MTX, MTX pré-biológico (MTX pre-bio, isto é antes do início de terapêutica com agentes biológicos), anti-TNF e TCZ. Para tal, amostras de sangue periférico foram colhidas a doentes ERA (n=13) e AR estabelecidas após tratamento com MTX (n=15) e MTX pre-bio (n=25). No grupo de doentes MTX pre-bio, foi efetuada uma segunda colheita de sangue a um grupo de doentes com AR após 8 meses de tratamento com anti-TNF (n=7) e a outro grupo após 6 meses de tratamento com TCZ (n=9). Amostras de sangue de controlos saudáveis (n=15) com a mesma proporção de idades e género foram também colhidas e processadas para comparação de resultados. Procedeu-se ao isolamento das células mononucleares do sangue periférico (linfócitos B e T, monócitos e granulócitos) e, posteriormente, separaram-se os linfócitos B. De seguida, a partir dos linfócitos B isolados com purezas acima dos 90%, extraiu-se o RNA, sintetizou-se o DNA complementar (cDNA) e fizeram-se ensaios de PCR em tempo real para estudar a expressão génica do grupo de genes acima mencionados.

Os resultados da expressão génica revelaram um aumento dos níveis de mRNA do BAFF-R no grupo de doentes com AR estabelecida tratados com MTX ($p=0.0212$) e anti-TNF ($p=0.0025$) relativamente aos controlos. Além disso, também se observou um aumento da expressão do BAFF-R no grupo de doentes tratados com anti-TNF quando comparado com os doentes ERA ($p=0.0006$). Por outro lado, não foram observadas diferenças significativas na expressão génica de TACI e BCMA em todos os grupos analisados. A análise da expressão génica dos *Toll-like receptors* revelou diferenças significativas nos grupos de doentes com AR estabelecida em comparação com os controlos. De facto, verificou-se um aumento da expressão de TLR7 e TLR10 no grupo de doentes com AR estabelecida tratados com anti-TNF em relação aos controlos ($p=0.0385$ e $p=0.0097$, respetivamente) e, além disso, foi também observado um aumento da expressão do gene TLR10 nos doentes tratados com anti-TNF quando comparados com os doentes ERA ($p=0.0801$). Adicionalmente, foi observado um aumento da expressão do gene TLR9 nos linfócitos B do grupo de doentes com AR estabelecida após tratamento com MTX relativamente aos controlos ($p=0.0043$). Verificou-se também uma sobreexpressão do gene B2M em todos os grupos de AR estabelecida quando comparados com os controlos: MTX ($p=0.0099$), MTX pre-bio ($p=0.0116$), anti-TNF ($p=0.0025$) e TCZ ($p=0.0112$), embora não tenham sido

detetadas diferenças significativas nos doentes ERA. Observou-se ainda que os níveis de expressão do gene BCL-2 estavam significativamente elevados em doentes com AR estabelecida após tratamento com MTX ($p<0.0001$), MTX pre-bio ($p<0.0001$), anti-TNF ($p=0.0014$) e TCZ ($p=0.0042$) não só em comparação com os controlos, mas também com os doentes ERA ($p<0.0001$). Além disso, verificou-se um aumento significativo nos níveis de expressão génica de CD32 nos doentes com AR estabelecida após tratamento com anti-TNF quando comparados com os controlos ($p=0.0043$) e com os doentes ERA ($p=0.0015$). Observou-se ainda um ligeiro aumento da expressão génica de CD32 em doentes com AR estabelecida após tratamento com MTX pre-bio relativamente aos controlos ($p=0.0487$). Contudo, foi observado um aumento significativo da expressão de CD32 neste grupo quando comparado com os doentes ERA ($p=0.0015$). Adicionalmente, não foram observadas diferenças significativas na expressão génica de CXCR5, AID e BLIMP-1 em todos os grupos analisados.

Por fim, quando se procedeu à análise da expressão génica antes e após o tratamento com anti-TNF e TCZ, não foram observadas quaisquer diferenças significativas para todos os genes estudados. Em relação aos dados clínicos dos doentes, verificou-se ainda que o tratamento com TCZ comparativamente com as outras terapêuticas estudadas, parece ser o mais eficaz a reduzir significativamente os valores séricos da proteína-C reativa, a velocidade de sedimentação e a actividade geral da doença indicada pelo DAS28. De salientar, no entanto, que não foi observada nenhuma correlação significativa entre a idade, os valores da proteína-C reactiva, a velocidade de sedimentação, o DAS28 e o número de articulações dolorosas e tumefactas com os resultados obtidos da expressão génica para todos os grupos estudados, independentemente do tratamento administrado aos doentes.

Com a realização deste estudo foi possível concluir que em doentes com AR estabelecida existem alterações na expressão de genes associados à ativação dos linfócitos B, quer através do recetor do BAFF (BAFF-R), quer através de TLRs (TLR7, TLR9, TLR10); e/ ou à inibição de linfócitos B (CD32), assim como perturbações na expressão de genes associados à inflamação (B2M) e apoptose (BCL-2). Nos doentes com AR estabelecida, o tratamento com anti-TNF e TCZ parece influenciar a expressão destes genes em comparação com os indivíduos saudáveis, embora não sejam detectadas diferenças significativas na expressão génica em relação à fase anterior ao início do tratamento. Além disso, a expressão génica em doentes não tratados com AR inicial com menos de 1 ano de duração da doença parece ser semelhante a dos indivíduos saudáveis. Estes resultados sugerem que a expressão génica de linfócitos B em doentes com AR sofre alterações em

diferentes fases de desenvolvimento da doença, podendo ser influenciada pelas opções terapêuticas.

Palavras-chave: Artrite Reumatóide, Linfócitos B, Expressão génica, Metotrexato, Anti-TNF, Tocilizumab

ABSTRACT

Rheumatoid arthritis (RA) is a systemic autoimmune disease that mainly affects the joints. The clinical success of B cell depletion therapy with rituximab in RA has reinforced the role of B cells in RA pathogenesis. Indeed, recent studies have shown that very early RA patients (with less than 6 weeks of disease duration) have disturbances in circulating memory B cells and increased levels of cytokines directly related with B cell activation and survival, which supports an active role of B cells in RA development since early disease onset.

The main goal of the present study was to analyze the expression of a group of genes directly related with B cell activation, maturation and survival in untreated early RA (ERA) (< 1 year of disease duration) and established RA patients after treatment with methotrexate (MTX), MTX pre-biologic (MTX pre-bio), TNF inhibitors and tocilizumab (TCZ) in order to compare the effects of the therapeutic options on B cell gene expression. For that, blood samples were collected from patients and age and sex-matched healthy donors. B cells were isolated, RNA extracted, cDNA synthesized and gene expression analysis performed by real time PCR.

We found that ERA patients have similar B cell gene expression levels when compared to healthy controls. However, increased gene expression was observed in BAFF receptor (BAFF-R), Toll-like receptors (TLR7, TLR9, TLR10), inhibition marker (CD32) and in genes associated with either active inflammation (B2M) or apoptosis (BCL-2) signaling pathways in established RA patients when compared to controls. No significant differences were observed in TACI, BCMA, CXCR5, AID and BLIMP-1 gene expression in all groups analyzed. Furthermore, the analysis of B cell gene expression levels in established RA patients after treatment with TNF inhibitors and TCZ in comparison with baseline values did not reveal significant differences.

Overall, these results suggest that the expression of genes related with B cell activation and survival are increased in later stages of RA development, which might be influenced by treatment options when compared to healthy individuals. Moreover, B cell gene expression in early RA patients does not seem to significantly change during the first year of RA development when compared to controls.

Keywords: Rheumatoid arthritis, B cells, Gene expression, Methotrexate, TNF inhibitors, Tocilizumab

INDEX

Acknowledgements	i
Resumo	ii
Abstract	vii
Index	viii
List of Abbreviations	x
List of Figures	xii
List of Tables	xiii
1. Rheumatoid Arthritis	1
1.1 Definition	1
1.2 Diagnostic and clinical assessment	1
1.3 Etiology and pathophysiology	3
1.4 Genetic factors and environment	5
1.5 Treatment	6
1.5.1 <i>Methotrexate</i>	7
1.5.2 <i>TNF antagonists</i>	7
1.5.3 <i>Tocilizumab</i>	7
1.5.4 <i>Rituximab</i>	8
2. B cells	9
2.1. B cell development	9
2.2. B cells and rheumatoid arthritis	9
3. Aims	11
4. Materials and methods	12
Patients	12
Isolation of peripheral blood mononuclear cells and B cell separation	12
RNA extraction and complementary DNA (cDNA) synthesis	12
Real-time quantitative polymerase chain reaction	13
Statistical analysis	13
5. Results	15
5.1. Clinical characterization of patients	15
5.2. Established RA, but not early RA patients have alterations in B cell gene expression levels in comparison with healthy controls	16
5.3. Treatment with TNF inhibitors and tocilizumab does not affect B cell gene expression	20

6. Discussion.....	23
7. Limitations and Future Perspectives.....	27
8. References.....	28

LIST OF ABBREVIATIONS

18S rRNA	18S ribosomal RNA
ACPA	Anti–citruUinated protein antibody
ACR	American College of Rheumatology
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand of the TNF family
BAFF	B cell activating factor of the TNF family
BCL-2	B-cell lymphoma 2
BCMA	B cell maturation antigen
Blimp-1	B lymphocyte-induced maturation protein
B_{reg}	Regulatory B cell
CRP	C-reactive protein
CpG	C-phosphate-G
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DAS28	Disease Activity Score in 28 joints
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FcγR	Fc-gamma receptor
Foxp3	Forkhead box P3
GC	Germinal center
GM-CSF	Granulocyte-macrophage colony stimulating factor
HCQ	Hidroxychloroquine
HLA	Human leucocyte antigen
Ig	Immunoglobulin
IL-	Interleukin
INF-γ	Interferon-gamma
MHC	Major histocompatibility complex
M-CSF	Macrophage colony-stimulating factor
MIP	Macrophage inflammatory protein
MCP	Monocyte chemoattractant protein
MTX	Methotrexate
MZ	Marginal zone

NF-κB	Nuclear factor-kappa B
PAD	Peptidylarginine deiminase
PTPN22	Protein tyrosine phosphatase non-receptor 22
RA	Rheumatoid Arthritis
RAG	Recombination activating gene
RANK	Receptor activator of nuclear factor-κB
RF	Rheumatoid factor
SE	Shared epitope
STAT-4	Signal transducer and activator of transcription protein 4
SSZ	Sulfasalazine
TACI	Transmembrane activator and calcium-modulator cyclophilin ligand interactor
TLR	Toll- like receptor
TCR	T cell receptor
TGF-β	Tumor growth factor β
TNF	Tumor necrosis factor
T_{Reg}	Regulatory T cell

LIST OF FIGURES

Fig. 1 – X-ray from a normal hand (left) and a hand from a patient with rheumatoid arthritis..	1
Fig. 2 - Pathogenesis of RA.....	3
Fig. 3 - Biologic agents and its targets.....	8
Fig. 4 - The multiple roles of B cells in RA	10
Fig. 5 - BAFF-R gene expression is significantly increased in B cells from established RA patients after treatment with MTX and TNF inhibitors..	17
Fig. 6 - Established RA patients have alterations in the gene expression levels of TLR7, TLR9 and TLR10 after treatment with MTX and/ or TNF inhibitors.	18
Fig. 7 - Established RA patients have increased gene expression levels of B2M, CD32 and BCL-2 irrespective of the treatment	19
Fig. 8 - ERA and established RA patients have normal gene expression levels of AID, BLIMP-1 and CXCR5.	20
Fig. 9 - B cell gene expression is not affected by TNF inhibitors in established RA when compared to baseline.	21
Fig. 10 - B cell gene expression is not affected by tocilizumab in established RA when compared to baseline	22

LIST OF TABLES

Table 1 - The ACR 1987 criteria for classification of RA	2
Table 2 - New classification criteria for rheumatoid arthritis	2
Table 3 - Primers used to examine the expression levels in pPCR	14
Table 4 - Clinical information of controls, ERA and established RA patients after treatment with MTX, MTX pre-bio, TNF inhibitors and TCZ	15

1. RHEUMATOID ARTHRITIS

1.1 Definition

Rheumatoid arthritis (RA) is a chronic, progressive, inflammatory disease that affects ~0.5%–1% of the adult population ¹. It is characterized by a peripheral polyarthritis that leads to deformity through the stretching of tendons and ligaments and destruction of joints through

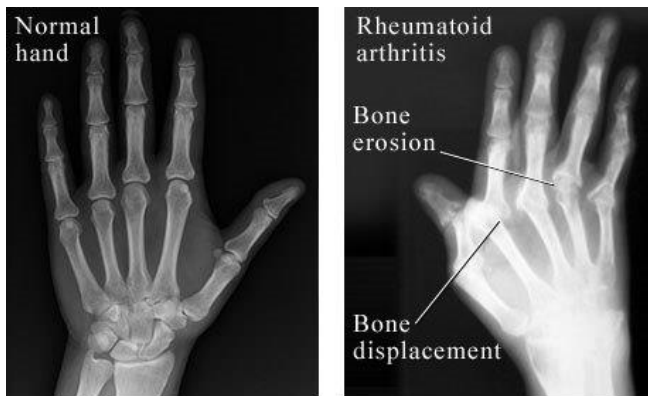


Fig. 1 – X-ray from a normal hand (left) and a hand from a patient with rheumatoid arthritis (Adapted from WebMD)

the erosion of cartilage and bone. If not treated, this autoimmune disease causes major disabilities which account for socioeconomic burden. Moreover, patients with RA have reduced life expectancy due to systemic manifestations ^{2,3}. Disease onset usually occurs between 30-50 years of age and is more frequent in women than men (ratio 3:1) ⁴.

The origin of RA is not associated to a single mechanism but it is known to involve a complex dysregulation in the immune system, genotype influence and environmental interactions. Thus, RA is presented as a heterogeneous condition mainly characterized by synovial inflammation and hyperplasia (swelling), cartilage and bone destruction, and systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders ^{5,6}.

1.2 Diagnostic and clinical assessment

For years, RA diagnosis was established according to the 1987 American College of Rheumatology (ACR) criteria (Table 1) ⁷. In 2010, the ACR and the European League Against Rheumatism (EULAR) developed a new criteria that allowed more specificity when distinguishing RA from other joint diseases and more sensitivity to identify individuals in the early stages of RA (Table 2) ^{8–10}.

The early stages of RA are mainly characterized by a symmetrical polyarthritis that affects the small joints of the hands and feet (metacarpophalangeal, proximal interphalangeal and metatarsophalangeal joints) before larger joints are affected ¹¹. Due to synovial hypertrophy, the joints become swollen, promoting pain and stiffness. Moreover, the levels of inflammatory markers in the blood, such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) raise ^{12,13}. Progression of joint damage in patients with RA is also highly correlated with overall levels of disease activity assessed by the disease activity score of 28 joints

(DAS28), which comprises the count of 28 tender or swollen joints in the hands, upper limbs and knees.¹⁴ The joints are also evaluated by imaging studies but these changes frequently take several years to develop¹⁵.

ACR 1987 criteria
<ol style="list-style-type: none"> 1. Morning stiffness (at least 1h) 2. Arthritis of three or more joint areas 3. Arthritis of hand joints (≥ 1 swollen joints) 4. Symmetrical arthritis 5. Rheumatoid nodules 6. Serum rheumatoid factor 7. Radiographic changes (erosions)
Four of these seven criteria must be present. Criteria 1–4 must have been present for at least 6 weeks

Table 1 - The ACR 1987 criteria for classification of RA (adapted from ⁶)

The presence of autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) (or anti-cyclic citrullinated peptides (anti-CCP)) in serum is also a characteristic of this autoimmune condition, although patients seronegative for

these antibodies also manifest the disease^{16,17}.

ACR/ EULAR 2010 criteria
<ol style="list-style-type: none"> 1. Joint involvement (0–5) <ul style="list-style-type: none"> • One medium-to-large joint (0) • Two to ten medium-to-large joints (1) • One to three small joints (large joints not counted) (2) • Four to ten small joints (large joints not counted) (3) • More than ten joints (at least one small joint) (5) 2. Serology (0–3) <ul style="list-style-type: none"> • Negative RF and negative ACPA (0) • Low positive RF or low positive ACPA (2) • High positive RV or high positive ACPA (3) 3. Acute-phase reactants (0–1) <ul style="list-style-type: none"> • Normal CRP and normal ESR (0) • Abnormal CRP or abnormal ESR (1) 4. Duration of symptoms (0–1) <ul style="list-style-type: none"> • Less than 6 weeks (0) • 6 weeks or more (1)
Points are shown in parenthesis. Cutpoint for rheumatoid arthritis 6 points or more. Patients can also be classified as having rheumatoid arthritis if they have: (a) typical erosions, (b) long-standing disease previously satisfying the classification criteria

Table 2 - New classification criteria for rheumatoid arthritis (Adapted from ⁶) RF = rheumatoid factor; ACPA= antibodies against citrullinated antigens; CRP= C-reactive protein; ESR = erythrocyte sedimentation rate

RF are autoantibodies that directly bind to the Fc portion of normal human IgG and ACPA are autoantibodies that recognize peptides or proteins containing citrulline, a non-standard

aminoacid generated by the post-translational modification of arginine by peptidylarginine deiminase (PAD) enzymes, in a process known as citrullination¹⁸. RF and ACPA can form immune complexes that deposit in the joints, activate complement and cause inflammation. The clinical usefulness of RF and ACPA has been acknowledged due to their good diagnostic sensitivity and prognostic value^{17,19}. Of note, RF and ACPA can develop several years before clinical onset¹⁶.

1.3 Etiology and pathophysiology

The etiology of RA is unknown. However, the development of this autoimmune disease seems to be a multistage process in which a number of genetic and environmental factors trigger the development of undifferentiated arthritis and a subsequent amount of stimuli are required for progression towards joint inflammation.

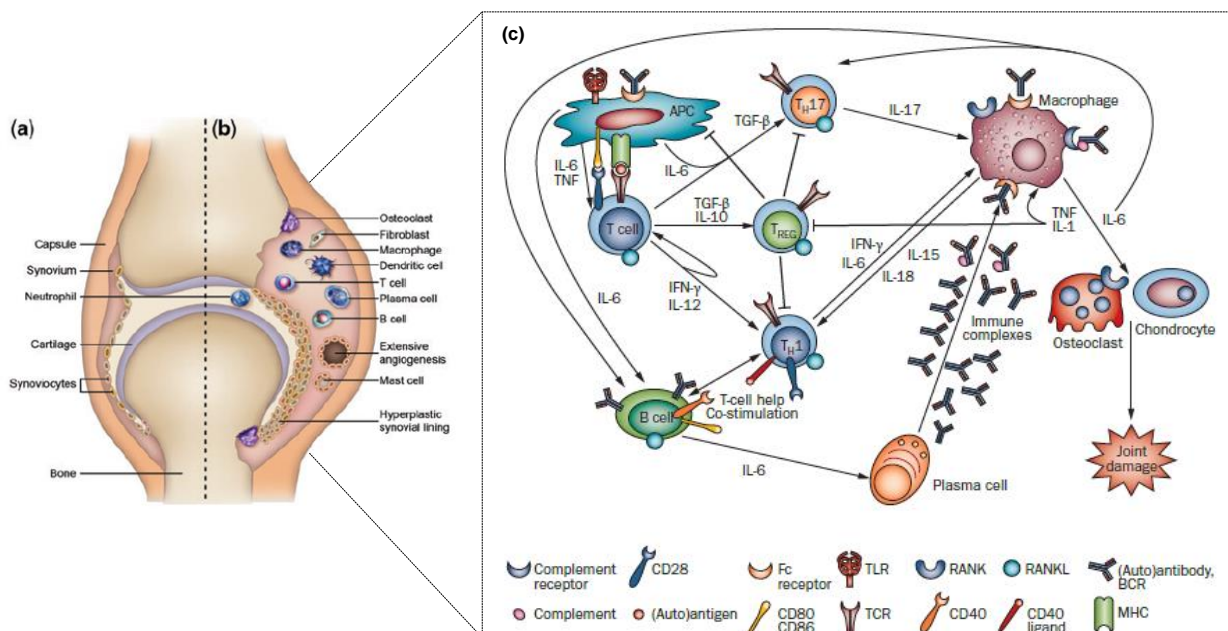


Fig. 2 - Pathogenesis of RA (Adapted from^{158,159}) **a)** represents the normal synovium; **b)** in RA the immune cells infiltrate the synovium making it hyperplastic with extensive angiogenesis; **c)** the adaptive and innate immune processes in the joint of RA

The synovial joint is composed of two adjacent bony ends each covered with a layer of cartilage, separated by a joint space and surrounded by the synovial membrane and joint capsule. The synovial lining (facing the cartilage and bone) consists of a thin layer of synoviocytes (macrophage and fibroblast-derived cells) that constitute the cellular part of the synovium. In healthy individuals, the synovium is almost an acellular structure (Fig. 2a)²⁰. Contrarily, in RA, a variety of mononuclear cells such as T cells, B cells, plasma cells, dendritic cells, macrophages and mast cells infiltrate the synovial membrane through the endothelia, forcing it to expand and become hyperplastic (Fig. 2b)²¹. Hyperplasia also results

from a marked increase in macrophage-like and fibroblast-like synoviocytes as they produce cytokines and factors that maintain inflammation by activating either themselves or their neighboring cells ²².

RA pathogenesis involves a complex network between cells from both innate and adaptive immune system (Figure 2c). An initial trigger activates macrophages in the synovial membrane, mediated locally through the action of antigen-activated CD4⁺ T helper cells ²³. The T cells invading the synovial membrane are primarily CD4⁺ memory cells, which produce interleukin-2 (IL-2) and interferon-gamma (IFN- γ), and are either already pre-activated or become further activated by antigen-presenting cells (APC) in conjunction with arthritogenic (auto)antigen(s) and appropriate MHC class-II molecules, co-stimulation (mainly through CD80/86 and CD28) and certain cytokines (IL-1, IL-15, IL-18) ²⁴. Activated T cells stimulate monocytes, macrophages and synovial fibroblasts. This will lead to an overproduction of pro-inflammatory cytokines, mainly TNF ²⁵, IL-1 ²⁶ and IL-6 ²⁷, which seem to constitute a key event leading to chronic inflammation ^{28–30}. TNF and IL-1 also induce receptor activator of nuclear factor- κ B (RANK) expression on macrophages that, after binding to RANK ligand (RANKL), stimulates macrophage differentiation into osteoclasts, which consequently resorb and destroy bone ³¹. Chondrocytes also become activated under the influence of IL-1 and IL-17A, leading to the release of metalloproteinases ⁵. Moreover, locally expressed degradative enzymes, serine proteases and aggrecanases, digest the extracellular matrix and destroy the articular structures ²⁰. An extensive angiogenesis process also occurs in RA, promoted by vascular endothelium growth factor (VEGF) production by monocytes, endothelial cells and synovial fibroblast in response to all the inflammatory stimuli, hypoxia and reduced apoptosis ³². This supports the formation and maintenance of pannus, a membrane of fibrovascular tissue that invades and destroys cartilage and bone ³³.

Recent studies also implicate IL-17-producing T cells (T_H17) as important effectors in RA pathogenesis due to the overexpression of IL-17 in the synovial joints, which is associated with an aggravation of inflammation and joint damage ^{34,35}. In fact, a neutrophil- and T_H17-driving cytokine pattern is present in the serum of untreated patients with very early RA ³⁶. Regulatory T cells (T_{Regs}) have also been detected in the synovium of RA patients with active disease and particularly in synovial fluid, but they seem to have impaired regulatory function ³⁷. The cytokine factors that sustain the expansion of T_{Regs} in the rheumatoid joint are not defined but probably include the regulatory cytokines IL-10 and TGF- β ³⁸.

B cells also have a relevant role in RA pathogenesis ³⁹, namely through autoantibody production (RF, ACPA), antigen presentation and cytokine release upon activation. Indeed, B cells produce RF and ACPA that form immune complexes that deposit in the joints. These immune complexes activate macrophages through Fc-gamma receptors (Fc γ R) that release

pro-inflammatory cytokines (TNF, IL-1, IL-6) ²³ that exacerbate the inflammatory process as previously described. Furthermore, it has been recently demonstrated that a cytokine pattern directly related with B cell activation and survival is present since the first weeks of RA onset, which supports an early intervention of B cells in RA development ⁴⁰.

1.4 Genetic factors and environment

RA comprises several genetic and environmental associations that are supposedly responsible for some alterations in post-transcriptional regulation ⁵. Twin studies have demonstrated a genetic role and showed that genetic factors contribute to 60% of risk of developing RA ⁴¹. The first established association was related with genes within the HLA (human leucocyte antigen) region, particularly the HLA-DRB1 allele that is associated with severe rheumatoid arthritis ^{42–44}. This gene encodes major histocompatibility complex (MHC) class-II β -chain molecules whose function is the presentation of antigen to CD4⁺ T cells. HLA-DRB1 alleles contain a common aminoacid motif termed “shared epitope” (SE) – a five aminoacid sequence motif in residues 70–74 of the HLA-DR β chain ⁴⁵. When antigens undergo post-translational modifications by citrullination (conversion of the aminoacid arginine to citrulline) this change allows antigens to fit in the HLA alleles that harbor this shared epitope which might result in breaking of tolerance that allows antibody formation against these antigens ^{46,47}. In fact, HLA class II alleles such as DR4 that form the ‘shared epitope’ are not primarily a risk factor for RA, but for the presence of ACPA ⁴⁸. A second association was established with the protein tyrosine phosphatase non-receptor 22 (*PTPN22*) gene, known to exert a negative feedback effect on the transmission of the signal generated by the T cell receptor (TCR) ^{5,32}. These genetic associations might explain the emergence of autoreactive responses in antigen presentation, T cell repertoire selection and alterations in peptide affinity ⁶. Other genes linked to RA susceptibility were also associated with the NF- κ B pathway (*REL*, *TRAF1*, and *TNFAIP3*) ⁴⁹ and other pathways related to T cell activation such as cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) that suppresses the interaction between T cells and antigen-presenting cells (APC); and signal transducer and activator of transcription protein 4 (*STAT-4*), responsible for transducing cytokine signals that regulate proliferation, survival and differentiation of lymphocytes ⁵⁰.

The environmental risk factors in RA include age and gender ¹⁸, a previous family history of RA ⁵¹, smoking ^{52–54}, obesity ⁵⁵, cardiovascular events ⁵⁶ and some infectious agents ^{57,58}. In fact, it has been documented that changes in the female hormonal environment such as pregnancy, breastfeeding and the use of the oral contraceptive might affect the development of RA ^{59–61}.

Some studies have also reported that the formation of immune complexes during exposure to some infectious agents might trigger the induction of RF autoantibodies, which have long been implicated in RA pathogenesis⁵⁸. Nonetheless, the most well-established environmental association with RA is smoking. Indeed, cigarette smoking has been associated with a consistently increased risk of developing RA, particularly in individuals that express the HLA-DRB1 SE alleles^{53,54}.

Overall, while RA susceptibility is genetically determined, disease onset may depend on non-genetic (i.e. environmental), epigenetic or post-translational events.

1.5 Treatment

Although no cure has been found for RA, it is clear that establishing a diagnosis as early as possible and immediate treatment are the basis for a successful management of these patients⁶². The main goals of RA treatment are the preservation of function and quality of life, remission of symptoms and control of systemic manifestations. Improvement in RA is defined by an outcome measure called ACR 20, ACR 50 or ACR 70, that is characterized as a reduction by 20, 50 or 70%, respectively, on the number of tender or swollen joints plus improvement in at least three of the five measures: pain, global assessment by the patient and levels of acute-phase reactants^{6,63}. Treatment options are divided in three main classes: 1) nonsteroidal anti-inflammatory drugs (NSAIDs); 2) corticosteroids; and 3) disease modifying anti-rheumatic drugs (DMARDs) (synthetic and biologic)⁶⁴. RA patients should begin their treatment with DMARDs, which not only relieve symptoms but also slow the progression of the disease. Often, DMARDs are prescribed along with NSAIDs and/or low-dose corticosteroids to decrease swelling and pain⁶. DMARDs have greatly improved the symptoms, function and quality of life of nearly all patients with RA. Synthetic DMARDs include methotrexate, leflunomide, sulfasalazine (SSZ) and hydroxychloroquine (HCQ), which are widely used in RA and have proven to be highly beneficial in decreasing inflammation and joint damage⁶⁴. Gold used to be also administered to RA patients, but nowadays is rarely prescribed since other drugs work better and/ or have fewer side effects⁶⁵. Nevertheless, patients still fail conventional DMARD therapy, maintaining a high disease activity and/ or suffering from severe adverse effects. In these cases, it is recommended that biologic DMARD therapy is initiated^{64,66}. Biologic DMARDs include TNF antagonists⁶⁷, which are sub-divided in neutralizing anti-TNF monoclonal antibodies (infliximab, adalimumab, golimumab, certolizumab) and a soluble TNF receptor-2–IgG-Fc fusion protein (etanercept)⁶⁸. Other biologic therapies include abatacept, a fusion protein composed of the Fc region of IgG1 fused to the extracellular domain of cytotoxic T-lymphocyte antigen (CTLA)-4 that binds to the CD80/ CD86 molecule and inhibits T cell activation⁶⁹, anakinra, an IL-1 receptor (IL-

1R) antagonist ²⁶; tocilizumab, an IL-6 receptor (IL-6R) blocking monoclonal antibody ⁷⁰ and rituximab, an anti-CD20 B-cell-depleting monoclonal antibody ⁷¹.

1.5.1 Methotrexate

Methotrexate (MTX) is an anti-metabolite and a folate analog designed to compete for folate receptors, which affects the *de novo* synthesis of purine and pyrimidine precursors of DNA and RNA ⁷². MTX also has anti-inflammatory effects at low doses ⁷³. MTX inhibits T-cell activation and proliferation, downregulates the expression of some activation and adhesion molecules, decreases immunoglobulin production, inhibits cyclooxygenases and lipooxygenases, and modulates the secretion of various cytokines by monocytes and macrophages. MTX, administered alone or in combination with either synthetic or biologic DMARDs remains the first drug of choice for patients with RA ⁷⁴.

1.5.2 TNF antagonists

TNF binds to two receptors, the type 1 TNF receptor (TNFR) (p55) and the type 2 TNFR (p75), that is expressed on many cell types, such as monocytes, T cells, B cells, NK cells, PMNs, mast cells, synovial fibroblasts and osteoblasts ²². TNF antagonists include neutralizing monoclonal antibodies such as infliximab, adalimumab or golimumab and a fusion protein, etanercept. Infliximab, is a chimeric IgG1 anti-TNF antibody containing the antigen-binding region of a mouse antibody and the constant region of a human antibody. It binds with high affinity to soluble and membrane-bound TNF, impairing the binding of TNF to its receptor. Infliximab also kills cells that express TNF through antibody-dependent and complement-dependent cytotoxicity (ADCC). Adalimumab and golimumab are recombinant human IgG1 monoclonal antibodies that bind to human TNF with high affinity, both impairing TNF binding to its receptors and lysing cells that express TNF on their surface. Etanercept, a soluble TNFR fusion protein composed of two dimers, each with an extracellular, ligand-binding portion of the higher-affinity type 2 TNFR (p75) linked to the Fc portion of human IgG1. This fusion protein binds to both TNF- α and TNF- β , thereby preventing each from interacting with its respective receptors. TNF antagonists are more effective when co-prescribed with MTX. Switching between anti-TNF treatments after secondary treatment failure can be associated with a sustained clinical improvement. Although usually effective, all anti-TNF treatments are associated with an enhanced risk of infections compared to non-biological DMARDs ⁶⁹.

1.5.3 Tocilizumab

IL-6 forms a complex by binding to IL-6R on the cell membrane, which then combines with gp130 that also resides on the cell membrane, forming a homodimer and giving rise to

intracellular signal transduction²⁷. Tocilizumab (TCZ) is a recombinant monoclonal antibody that inhibits the induction of biological activity due to IL-6 in cells that express both membrane-bound IL-6R and gp130 molecules, and also inhibits the induction of biological activity due to IL-6/IL-6R complex formation in cells that express gp130 alone⁷⁵. Inhibition of IL-6 significantly has showed improved signs and symptoms of RA and normalized the acute-phase reactants⁷⁶.

1.5.4 Rituximab

CD20 is expressed almost exclusively by B cells at different stages of differentiation, but it is not expressed by stem cells or by earlier precursors (i.e. pro-B cells), or by terminally differentiated plasma cells⁷⁷. Rituximab (RTX) is a monoclonal antibody that selectively depletes B cells by directly binding to CD20⁷⁸. RTX depletes B cells by inducing cell lysis, which can be mediated by complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity or apoptosis^{79,80}. The efficacy of B cell depletion therapy with RTX in RA patients has reinforced the relevance of B cells in RA pathogenesis^{81–83}. Importantly, RTX is generally well tolerated, with low risk of infections.

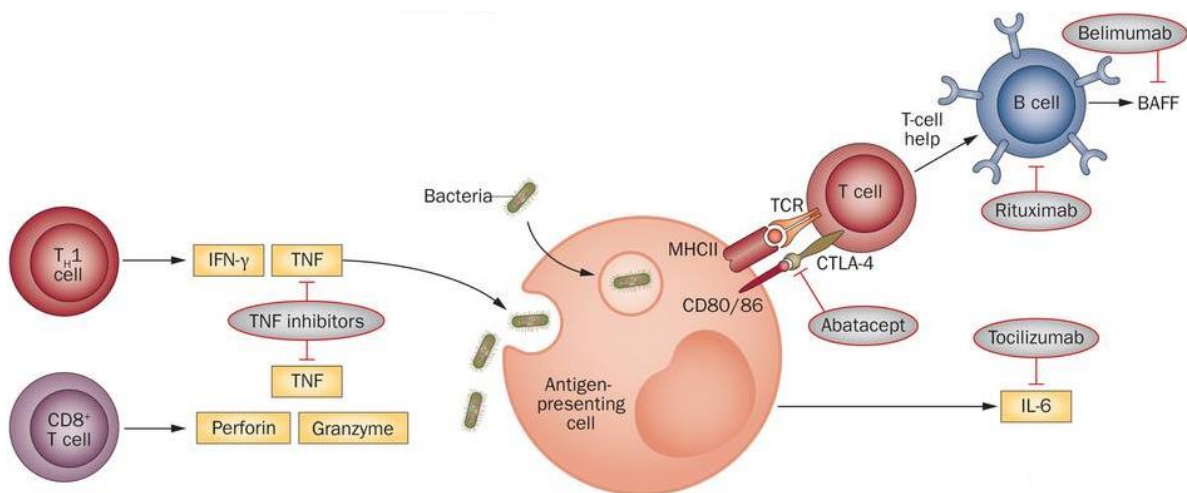


Fig. 3 - Biologic agents and its targets (Adapted from⁸⁴)

2. B CELLS

2.1. B cell development

In adults, B cell development starts in the bone marrow. The B cell progenitor (pro-B cell) is the earliest distinctive B-lineage maturation stage, characterized by recombination activating gene (RAG)-mediated V, D and J region rearrangement of the immunoglobulin heavy-chain locus. Pro-B cells proliferate in the bone marrow and differentiate into precursor B cells (pre-B cells), characterized by the expression of the cell surface pre-B-cell receptor (BCR). The bone marrow stages of human B-cell development are characterized by the expression of CD19 by pro-B cells and the subsequent expression of CD20 by pre-B cells, the rearrangement of the heavy-chain and light-chain immunoglobulin (Ig) loci, and the expression of cell surface IgM. In bone marrow, autoreactive B cells are eliminated through apoptosis or undergo immunoglobulin receptor editing⁸⁵. Some evidence suggest that these tolerogenic mechanisms can be compromised during autoimmune processes⁸⁶. Once IgM and IgD surface expression are fully achieved, these immature B cells leave the bone marrow into circulation as naïve B cells. These naïve B cells, which have never encountered an antigen, circulate in the blood and lymphatic systems and are carried to secondary lymphoid organs (lymph nodes, spleen, Peyer's Patches), where they further differentiate. In the presence of antigens, naïve B cells are stimulated via T-dependent and independent pathways in germinal centers (GC) and in the marginal zones (MZ) of lymphoid organs and differentiate into memory B cells or plasma cells^{78,85,87}.

2.2. B cells and rheumatoid arthritis

For years, RA was considered a T-cell driven disease^{88,89}. However, the disappointing results obtained with anti-CD4 therapy in humans reinforced the notion that perhaps other parts of the inflammatory process of RA should be better studied and understood^{90,91}.

B cell activity has become a subject of research not only because of the clinical success of B cell depletion therapy with rituximab^{81,83}, but also because the breakdown of tolerance mechanisms that normally regulate B cell development has been associated with autoimmunity^{92,93}. Importantly, recent studies have shown that very early RA patients (with less than 6 weeks of disease duration) have disturbances in circulating memory B cells⁹⁴ and increased levels of cytokines related with B cell activation and survival^{36,40}, which supports an active role of B cells in RA pathogenesis since early disease onset. Furthermore, histological studies have demonstrated that B cells highly infiltrate the synovial membrane, particularly memory B cells and plasma cells^{95,96}. Of note, the lymphocyte infiltrate in the synovium comprises various patterns of structural organization in which B cells are present: cells can be diffusely distributed, loosely aggregated or form ordered structures that contain

GCs (occurring in <20% of individuals affected with RA). The formation of ectopic GCs may contribute to the development of emerging self-reactive B cells as a result of local affinity maturation and receptor editing. Indeed, their presence may predispose to poorer outcome⁹⁷.

B cells can contribute to RA pathogenesis by several mechanisms (see figure 4):

- 1) *Autoantibody production*, such as RF and ACPA, to various synovium-derived antigens such as type II collagen, fibrinogen and filaggrin among others.
- 2) *Antigen presentation and T cell activation*. B cells can act as efficient APCs and stimulate T cells, allowing the development of memory T-cell subpopulations⁹⁸. Furthermore, T cell response in RA synovitis and ectopic lymphoid organization is B cell dependent⁹⁹. RF⁺ B cells are believed to play an important role in antigen presentation. In fact, it has been demonstrated that they can take up antigen–antibody immune complexes through their membrane Ig receptors, which have RF specificity¹⁰⁰.
- 3) *Cytokine and chemokine secretion*. B cells sequentially stimulated through the BCR and CD40 proliferate and secrete TNF, lymphotoxin- α and IL-6 that amplify the ongoing immune response¹⁰¹.

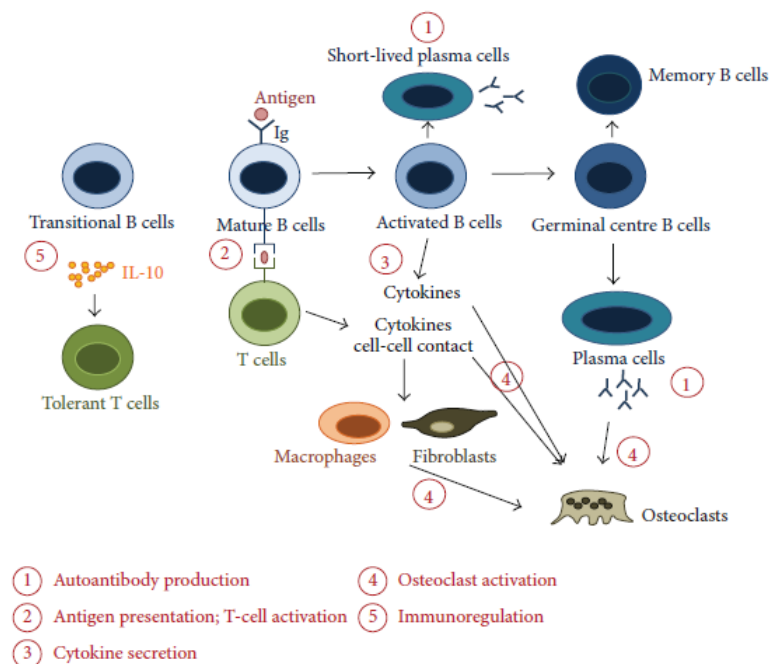


Fig. 4 - The multiple roles of B cells in RA (Adapted from¹⁰²) Immune responses potentially orchestrated by B cells in rheumatoid arthritis. B-T cell interactions result in the activation and differentiation of plasma cells, responsible for the production of autoantibodies (1). In turn, activated B cells provide help to T cells and induce differentiation of effector T cells that produce proinflammatory cytokines (2). B cells can also impact on other immune and nonimmune cell functions through secretion of cytokines, such as interleukin (IL)-1, IL-6, TNF- α , and IL-17A (3). Proinflammatory cytokines and receptor activator of nuclear factor κ B ligand (RANKL) produced by activated B cells, T cells, macrophages, and synovial fibroblasts promote the differentiation and activation of osteoclasts, leading to bone resorption (4). Further participation of B cells in bone homeostasis is suggested by the recognition that autoantibodies recognizing citrullinated vimentin are able to promote the differentiation of mononuclear cells to osteoclasts (4). B cells can also be immunoregulatory through the provision of IL-10 and other mechanisms yet to be elucidated (5).

4) *Osteoclast activation*. B cell survival and activation signals are mainly provided by TNF-superfamily members. B cells represent also a major source of RANKL in the rheumatoid environment, suggesting their direct involvement in the process of osteoclastogenesis and, consequently, bone erosion ¹⁰³.

5) *Immunoregulation*. Recent studies indicate the presence of a subset of B cells – B regulatory cells (B_{reg}) – specifically induced under inflammatory conditions that are capable of suppressing inflammation and/or enhancing the recovery process ¹⁰⁴. B_{reg} can act through: a) IL-10 production, which restores Th1/Th2 balance and directly inhibits inflammatory cascades; b) TGF-β1 production, which induces apoptosis of effector T cells; c) suppression of activated CD4⁺ T cells and d) interaction with other immune cells either directly or through secreted antibodies (reviewed in ³⁸).

3. AIMS

The main goals of the present study were:

- I. To analyze the expression of a group of genes directly related with B cell activation, maturation and survival in untreated early RA and established treated RA patients
- II. To compare the effects of treatment options (methotrexate - MTX; TNF antagonists and Tocilizumab - TCZ) on B cell gene expression in established RA patients

4. MATERIALS AND METHODS

Patients

Blood samples were collected from 13 consecutive patients with untreated polyarthritis (Rheumatology Department, Hospital de Santa Maria, Lisbon) of ≤ 1 year disease duration. After a minimum follow-up of 3 months, the patients fulfilled the 2010 ACR/ EULAR criteria for RA and were classified as early RA (ERA). In addition, blood samples from 15 patients with established RA treated with methotrexate (MTX); 25 patients with established RA treated with MTX before starting biologic DMARD therapy (MTX pre-biologic, MTX pre-bio); 7 patients under treatment with TNF inhibitors and 9 patients under tocilizumab (TCZ) treatment were also collected for comparison (Rheumatology Department, Hospital de Santa Maria, Lisbon). Furthermore, blood samples from 15 healthy donors were also collected and processed for comparison.

This study was approved by the local ethics committee (Comissão de Ética do Hospital de Santa Maria), and all patients and healthy donors signed an informed consent form. Patient care was conducted in accordance with standard clinic practice, and the study was performed in accordance with the Declaration of Helsinki (2008).

Isolation of peripheral blood mononuclear cells and B cell separation

Peripheral blood mononuclear cells (PBMC) were isolated from 60 ml heparinized whole blood following density gradient centrifugation with Ficoll (Biowest, France). Cellular counts were estimated with 0.4% Trypan Blue (Sigma-Aldrich, USA) in a Neubauer's chamber. B cells were isolated by positive MACS Separation using CD19 Microbeads and LS Columns (Miltenyi Biotec GmbH, Germany), according to the manufacturer's instructions. Purity of isolated B cells was analysed by flow cytometry using fluorochrome-conjugated CD20 FITC (BD Biosciences, USA) and CD3 APC (eBioscience, USA) antibodies. A total of 20000 cells/sample were acquired with LSR Fortessa (BD Biosciences, USA). The remaining B cells were stored at -80°C in RLT Buffer (Qiagen, Germany) supplemented with RNase inhibitor β -mercaptoethanol (1:100) until further use.

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from B cells using the RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions and treatment with RNase-free DNase Set (Qiagen, Germany) was performed to avoid contamination of genomic DNA. RNA

concentration and purity were determined with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Total RNA was reverse-transcribed into cDNA using DyNAmo™ cDNA Synthesis Kit for qRT-PCR (Finnzymes, Finland) with Moloney murine leukemia virus (M-MuLV) reverse transcriptase, random hexamers (300 ng/μl) and 2X RT Buffer, according to the manufacturer's instructions, performed on Piko Thermal Cycler (Finnzymes, Finland). The cDNA samples were stored at -20°C.

Real-time quantitative polymerase chain reaction

The expression of a group of genes directly related with B cell activation, maturation and survival was assessed by real-time quantitative polymerase chain reaction (qPCR) performed on Rotor-Gene 6000 (Corbett Life Science, USA) using SensiMix SYBR No-ROX Kit (Bioline, United Kingdom). The qPCR program consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. Genes and primer sequences analyzed in this study are indicated in Table 3. Primers were designed using the National Center for Biotechnology Information (NCBI)/ Primer-BLAST. The 18S ribosomal RNA (18S rRNA) was used as endogenous controls in relative quantification using the standard curve method. All data were analyzed with Rotor-Gene 6000 Series Software.

Statistical analysis

Statistical differences were determined with GraphPad Prism (GraphPad, San Diego, USA). For populations that did not follow a Gaussian distribution, nonparametric tests were used. The Mann-Whitney test was used for comparisons between 2 independent groups. For comparisons between 3 or more groups, the Kruskal-Wallis and Dunn's multiple comparison tests were used. The Wilcoxon matched pairs test was used for comparisons between 2 paired groups. Correlation analysis was performed using Spearman's test. Differences were considered statistically significant for $p < 0.05$.

Table 3 - Primers used in real time PCR assay

GENE	NAME	SEQUENCES
18S rRNA	18S ribosomal RNA	Fw: 5'-GGA GTA TGG TTG CAA AGC TGA-3' Rv: 5'-ATC TGT CAA TCC TGT CCG TGT-3'
BAFF-R	BAFF receptor	Fw: 5'-CTG GTC CTG GTG GGT CTG-3' Rv: 5'-ACC TTG TCC AGG GGC TCT-3'
TACI	Transmembrane activator and	Fw: 5'-AGG CTC AGA AGC AAG TCC AG-3' Rv: 5'-CCA GGA AGC AGC AGA GGA-3'
BCMA	B cell maturation antigen	Fw: 5'-AGG ACG AGT TTA AAA ACA CAG GA-3' Rv: 5'-TCA CAG GTG CAT TCT TCC AC-3'
CXCR5	C-X-C chemokine receptor 5	Fw: 5'-GGA GCC TCT CAA CAT AAG ACA GT-3' Rv: 5'-ATT TTC CAC CAG GGA GGT GTC-3'
AID	Activation-induced cytidine deaminase	Fw: 5'-GGA CTT TGG TTA TCT TCG CAA T-3' Rv: 5'-GTC GGG CAC AGT CGT AGC-3'
BLIMP-1	B lymphocyte-induced maturation protein	Fw: 5'-ACG TGT GGG TAC GAC CTT G-3' Rv: 5'-CTG CCA ATC CCT GAA ACC T-3'
β2M	β2 – Microglobulin	Fw: 5'-CTA TCC AGC GTA CGC CAA AGA TTC-3' Rv: 5'-CTT GCT GAA AGA CAA GTC TGA ATG-3'
BCL-2	B-cell lymphoma 2	Fw: 5'-TTG ACA GAG GAT CAT GCT GTA CTT-3' Rv: 5'-ATC TTT ATT TCA TGA GGC ACG TT-3'
FCGR2B (CD32)	FcγReceptor II B	Fw: 5'-GTG CTA TTC CTG GCT CCT GTT-3' Rv: 5'-CGT GTG GGT GGG AAT GAG AT-3'
TLR7	Toll-like receptor 7	Fw: 5'-CAC CTC TCA TGC TCT GCT CTC-3' Rv: 5'-TCT AGC CCC AAG GAG TTT GGA-3'
TLR9	Toll-like receptor 9	Fw: 5'-GGA CCT CGA GTG TGA AGC AT-3' Rv: 5'-TGG AGC TCA CAG GGT AGG AA-3'
TLR10	Toll-like receptor 10	Fw: 5'-TCT GCT GAG AGA GTG CAA GC-3' Rv: 5'-ACA TGT TGG AGC AGT TGG TCA-3'

5. RESULTS

5.1. Clinical characterization of patients

A group of untreated polyarthritis patients (n=13) with less than 1 year of disease duration and classified as early RA (ERA) according to the 2010 ACR/ EULAR criteria after a minimum follow-up of 3 months was included in this study. ERA patients had a mean \pm standard deviation age of 58 ± 14 years old, 85% were female, 69% were RF positive, 62% were anti-CCP positive and DAS28 score was 4.1 ± 2.0 . A group of established RA patients under MTX treatment (n=15) with a mean age of 55 ± 14 years old, 87% female and a DAS28 of 2.7 ± 1.2 was also included. Furthermore, a group of established RA patients under MTX treatment pre-biological therapy (MTX pre-bio, n=25) with a mean age of 58 ± 12 years old, 88% female and a DAS28 of 5.2 ± 1.5 was also analyzed. From the MTX pre-bio group, a second blood collection was performed to RA patients that had either initiated treatment with TNF-inhibitors (n=7) or with tocilizumab (TCZ) (n=9), after an average follow-up of 8 and 6 months of treatment, respectively. In addition, blood samples were collected from age and sex-matched healthy donors (n=15). The clinical information and data from all patients and healthy controls included in this study is indicated in Table 4.

	Controls (n=15)	ERA (n=13)	MTX (n=15)	MTX pre-bio (n=25)	TNF (n=7)	TCZ (n=9)
Age (years)	50 \pm 7	58 \pm 14	55 \pm 14	58 \pm 12	52 \pm 16	61 \pm 13
Sex (% female)	73	85	87	88	80	100
Disease duration (years)	NA	≤ 1	7 \pm 6	11 \pm 8	6 \pm 3	12 \pm 7
CRP (mg/dl)	ND	1.5 \pm 2.1	0.5 \pm 0.6	1.4 \pm 3.1	1.2 \pm 1.1	0.2 \pm 0.3#
ESR (mm/1st hour)	ND	42 \pm 25	23 \pm 23	29 \pm 16	44 \pm 47	4 \pm 2**#&
VAS	NA	40 \pm 33	42 \pm 33	69 \pm 18	66 \pm 27	67 \pm 10
DAS28	NA	4.1 \pm 2.0	2.7 \pm 1.2#	5.2 \pm 1.5	4.4 \pm 1.3	2.9 \pm 1.0#
Swollen joints	NA	3 \pm 5	1 \pm 2#	6 \pm 6	3 \pm 3	1 \pm 1
Tender joints	NA	5 \pm 5	2 \pm 3#	12 \pm 8	7 \pm 4	2 \pm 3#
RF (+) %	ND	69	87	75	60	86
ACPA (+) %	ND	62	60	78	40	86

Table 4 - Clinical information of controls, ERA and established RA patients after treatment with MTX, MTX pre-bio, TNF inhibitors and TCZ. ERA - Early Rheumatoid Arthritis; RA - Rheumatoid Arthritis; MTX - Methotrexate; MTX pre-bio - Methotrexate pre-biologic; TNF - TNF inhibitors; TCZ - Tocilizumab; CRP - C-reactive protein; ESR - Erythrocyte Sedimentation Rate; VAS - Visual Analogue Scale; DAS28 - Disease Activity Score of 28 joints; RF - Rheumatoid Factor; ACPA- anti-cyclic citrullinated peptide; NA - not applicable; ND - not determined. Values are represented as mean \pm standard deviation. (** $p < 0.05$ in comparison with ERA; # $p < 0.05$ in comparison with MTX pre-bio; & $p < 0.05$ in comparison with TNF)

5.2. Established RA, but not early RA patients have alterations in B cell gene expression levels in comparison with healthy controls

In this study, a group of genes related with B cell homeostasis and survival (BAFF-R, TACI, BCMA), class-switching (AID), chemotaxis (CXCR5), plasma cell differentiation (BLIMP-1), immune system activation (B2M), apoptosis (BCL-2), B cell inhibition (CD32) and activation through Toll-like receptors (TLR7, TLR9, TLR10) were analyzed in untreated ERA and established RA patients under treatment with MTX, MTX pre-bio, TNF inhibitors and TCZ. Results were compared with age and sex-matched healthy controls.

Since some of the studied genes are also expressed by monocytes and T cells, isolating B cells from PBMC samples was mandatory. Therefore, the purity of B cell isolation was tested for each sample by flow cytometry and samples were excluded if the purity was under 90%. Additionally, samples were also excluded if the RNA concentration obtained from isolated B cells was too low to perform a gene expression analysis of all genes of interest.

It was found that untreated ERA patients (≤ 1 year of disease duration) have similar B cell gene expression levels with no statistically significant differences when compared to healthy controls for all genes analyzed (Figures 5-8). However, established RA patients had alterations in B cell gene expression levels in comparison with controls.

The analysis of all BAFF receptors has revealed that BAFF-R gene expression was significantly increased in established RA patients after treatment with MTX ($p=0.0212$) and TNF inhibitors ($p=0.0025$) in comparison with the control group (Figure 5). Furthermore, BAFF-R gene expression was also significantly increased in established RA patients after treatment with TNF inhibitors when compared to ERA ($p=0.0006$). Nevertheless, no statistically significant differences were detected in TACI and BCMA gene expression levels in all groups studied.

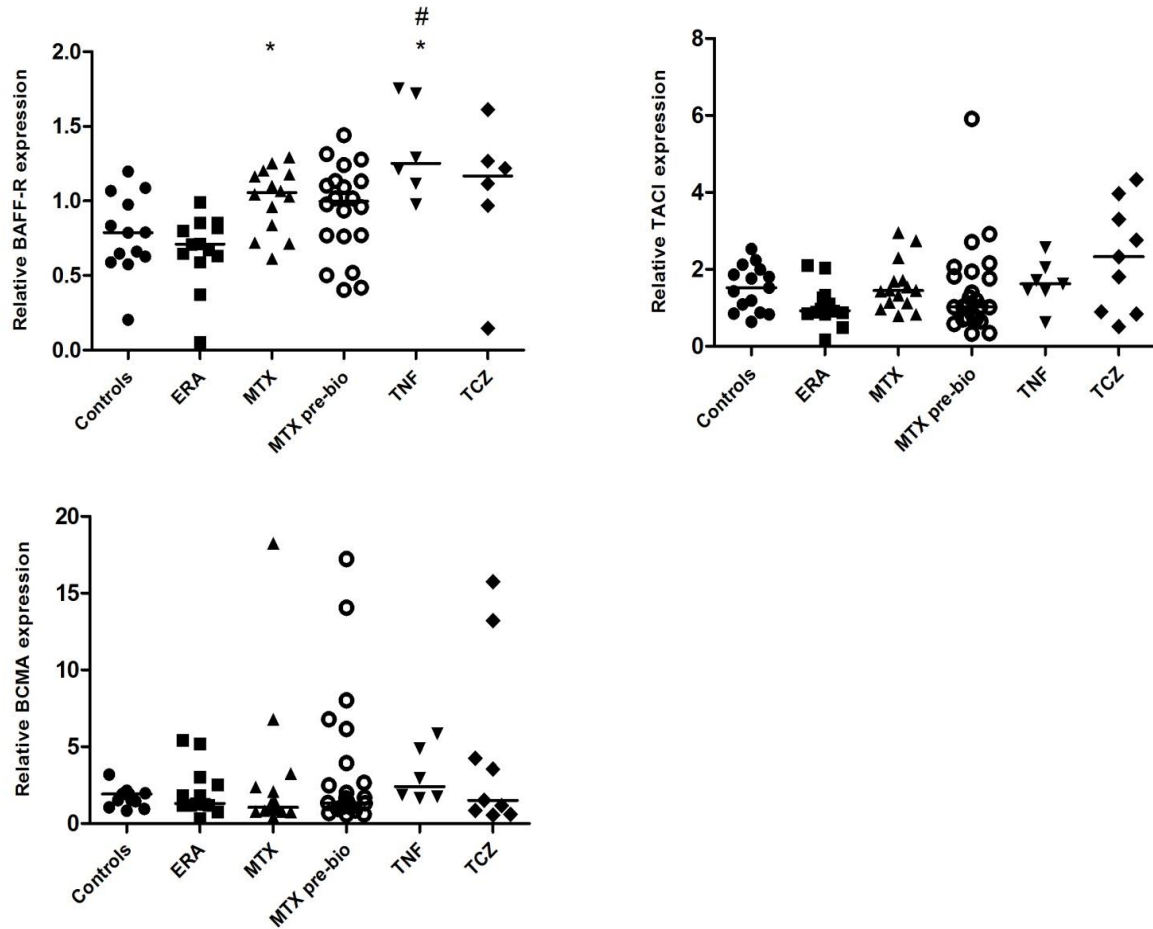


Fig. 5 - BAFF-R gene expression is significantly increased in B cells from established RA patients after treatment with MTX and TNF inhibitors. A nonparametric statistical analysis was performed with Kruskal-Wallis and Mann-Whitney tests: bars represent median relative gene expression values. * $p < 0.05$ in comparison with Controls; # $p < 0.05$ in comparison with ERA.

Alterations in the B cell gene expression levels of TLR7, TLR9 and TLR10 were also observed in established RA patients (Figure 6). Indeed, it was found that TLR7 and TLR10 gene expressions were significantly increased in established RA patients after treatment with TNF inhibitors when compared to controls ($p=0.0385$ and $p=0.0097$, respectively). Furthermore, TLR10 gene expression levels were also significantly increased in established RA under anti-TNF therapy when compared to ERA patients ($p=0.0801$). Moreover, TLR9 gene expression was significantly increased in established RA patients under MTX treatment ($p=0.0043$) when compared to controls. No other statistically significant differences were detected in all groups analyzed.

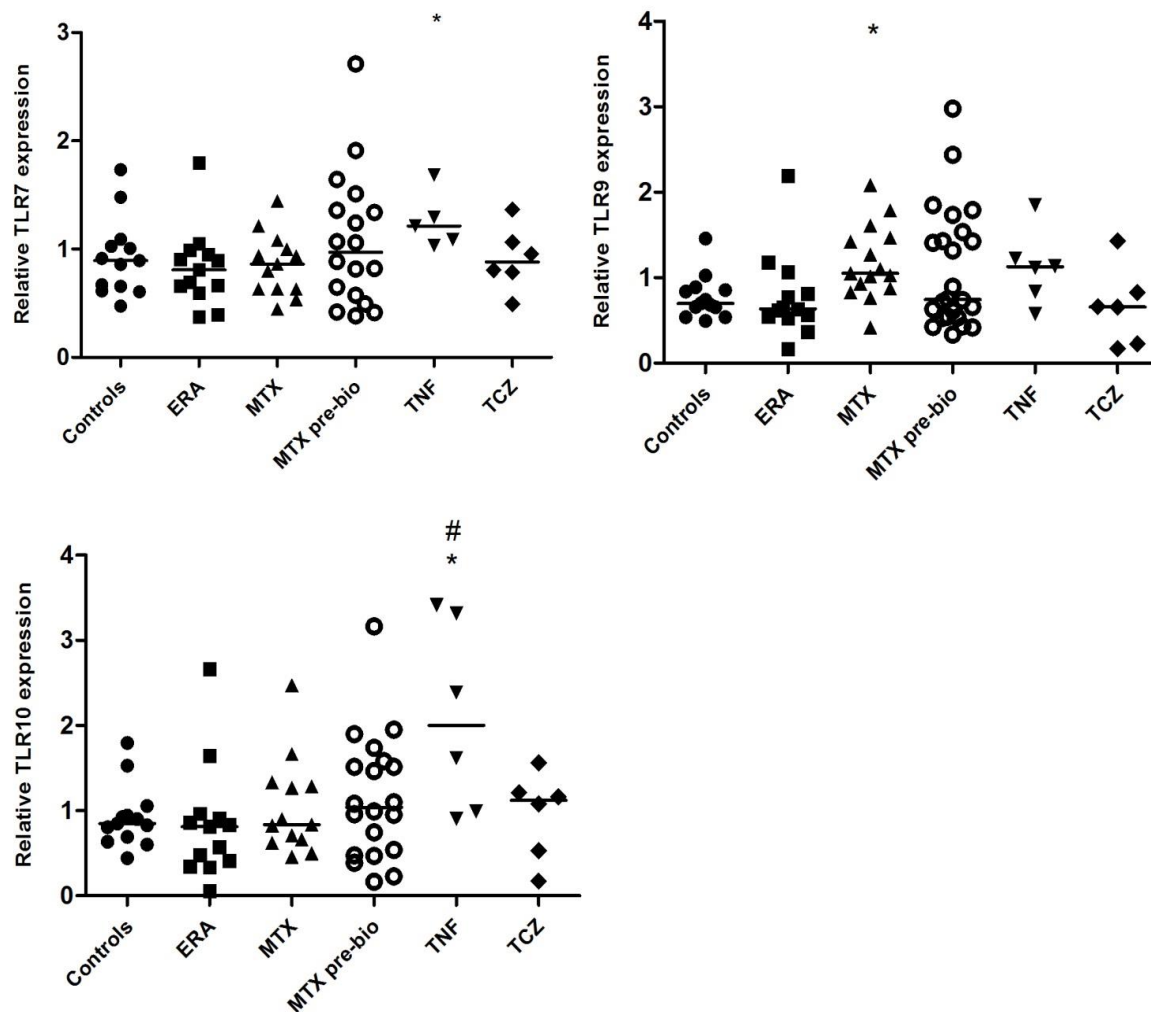


Fig. 6 - Established RA patients have alterations in the gene expression levels of TLR7, TLR9 and TLR10 after treatment with MTX and/ or TNF inhibitors. A nonparametric statistical analysis was performed with Kruskal-Wallis and Mann-Whitney tests: bars represent median relative gene expression values. * $p < 0.05$ in comparison with Controls; # $p < 0.05$ in comparison with ERA.

The analysis of the gene expression levels of B2M, an inflammatory marker, and CD32 (FCGR2B), an inhibitory B cell marker, also revealed disturbances in established RA patients (Figure 7). It was observed that B2M gene was overexpressed in B cells from established RA patients treated with MTX ($p=0.0099$), MTX pre-bio ($p=0.0116$), TNF inhibitors ($p=0.0025$) and TCZ ($p=0.0112$) when compared to controls, but no significant differences were detected in ERA patients in comparison with controls. The gene expression levels of CD32 were significantly elevated in established RA patients treated with TNF inhibitors in comparison not only with controls ($p=0.0043$), but also with ERA patients ($p=0.0015$). Furthermore, a tendency for increased CD32 gene expression levels was also observed in established RA patients treated with MTX pre-bio when compared to controls ($p=0.0487$). Nonetheless,

CD32 gene expression was significantly increased in established RA patients treated with MTX pre-bio when compared to ERA patients ($p=0.0015$).

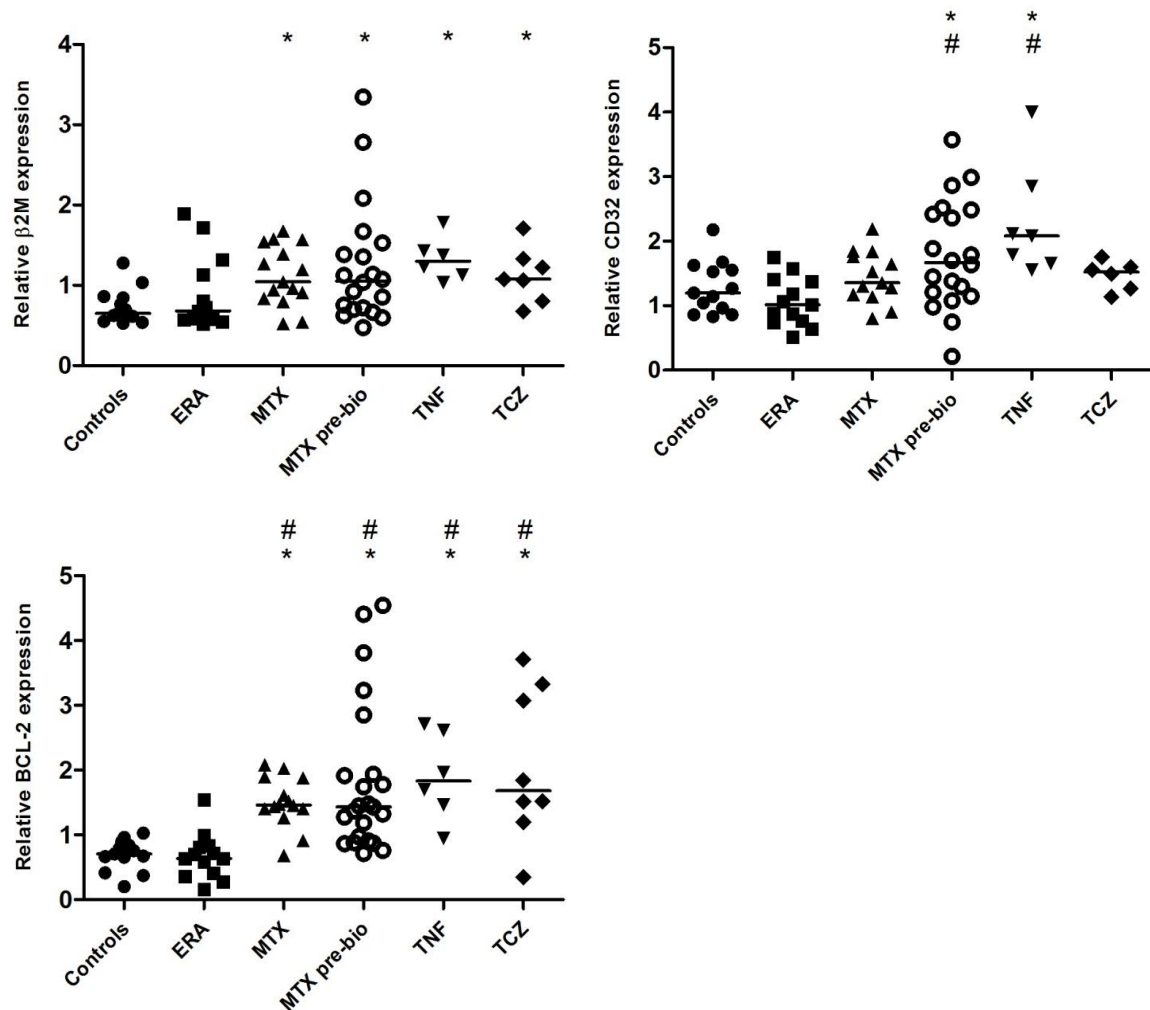


Fig. 7 - Established RA patients have increased gene expression levels of B2M, CD32 and BCL-2 irrespective of the treatment. A nonparametric statistical analysis was performed with Kruskal-Wallis and Mann-Whitney tests: bars represent median relative gene expression values. * $p < 0.05$ in comparison with Controls; # $p < 0.05$ in comparison with ERA.

In addition, BCL-2 gene was overexpressed in B cells from established RA patients treated with MTX ($p<0.0001$), MTX pre-bio ($p<0.0001$), TNF inhibitors ($p=0.0014$) and TCZ ($p=0.0042$) in comparison with controls and also with ERA patients ($p<0.0001$) (Figure 7).

It was also observed that ERA and established RA patients (irrespective of treatment) had similar AID, BLIMP-1 and CXCR5 gene expression levels when compared to healthy controls and no significant differences were detected (Figure 8).

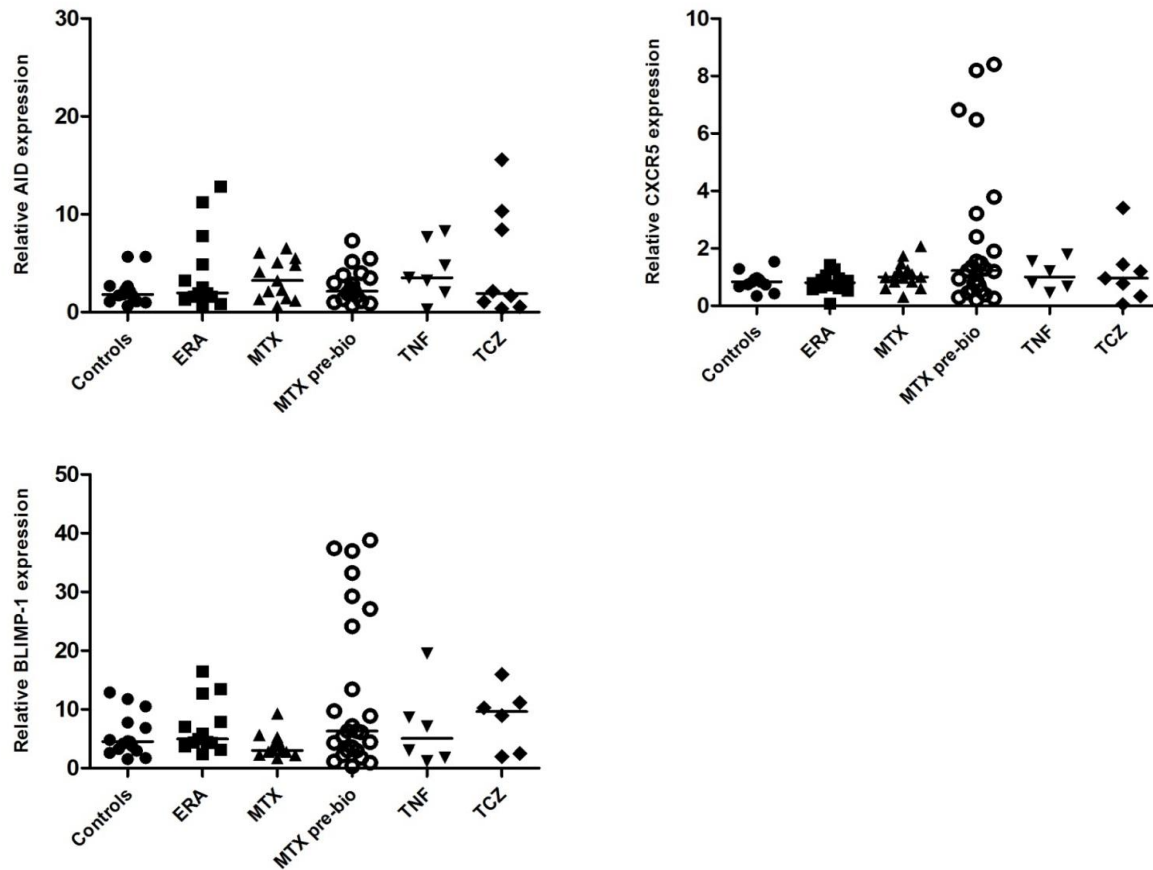


Fig. 8 - ERA and established RA patients have normal gene expression levels of AID, BLIMP-1 and CXCR5. No significant differences were observed in B cell gene expression levels of AID, BLIMP-1 and CXCR5 in all groups of patients when compared to healthy controls.

5.3. Treatment with TNF inhibitors and tocilizumab does not affect B cell gene expression

In order to understand the effect of treatment options before and after RA patients initiate treatment, a second blood collection was performed in established RA patients from MTX pre-bio group after a mean period of 8 months of treatment with TNF inhibitors (n=5) or after a mean of 6 months of treatment with TCZ (n=7). B cell gene expression levels were analyzed after treatment and compared to baseline values. Nevertheless, no statistically significant differences were observed in the gene expression levels of all genes studied in established RA patients after treatment with TNF inhibitors (Figure 9) or TCZ (Figure 10).

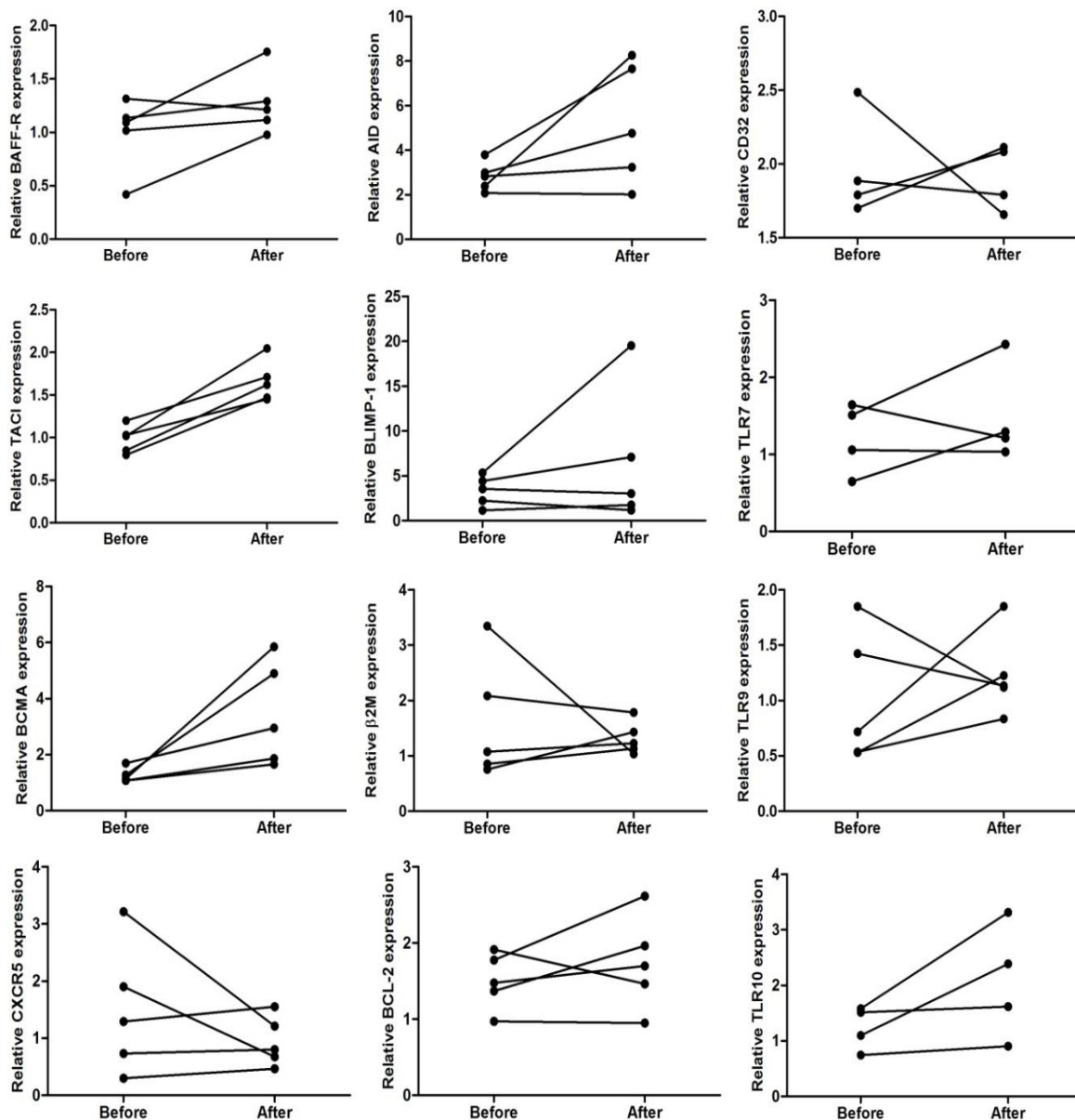


Fig. 9 - B cell gene expression is not affected by TNF inhibitors in established RA when compared to baseline. B cell gene expression levels of BAFF-R, TACI, BCMA, CXCR5, AID, BLIMP-1, β 2M, BCL-2, CD32, TLR7, TLR9 and TLR10 were analyzed in established RA patients after 8 months of treatment with TNF inhibitors (n=5) and compared to baseline levels. No significant differences were observed in all B cell gene expression levels before and after anti-TNF therapy.

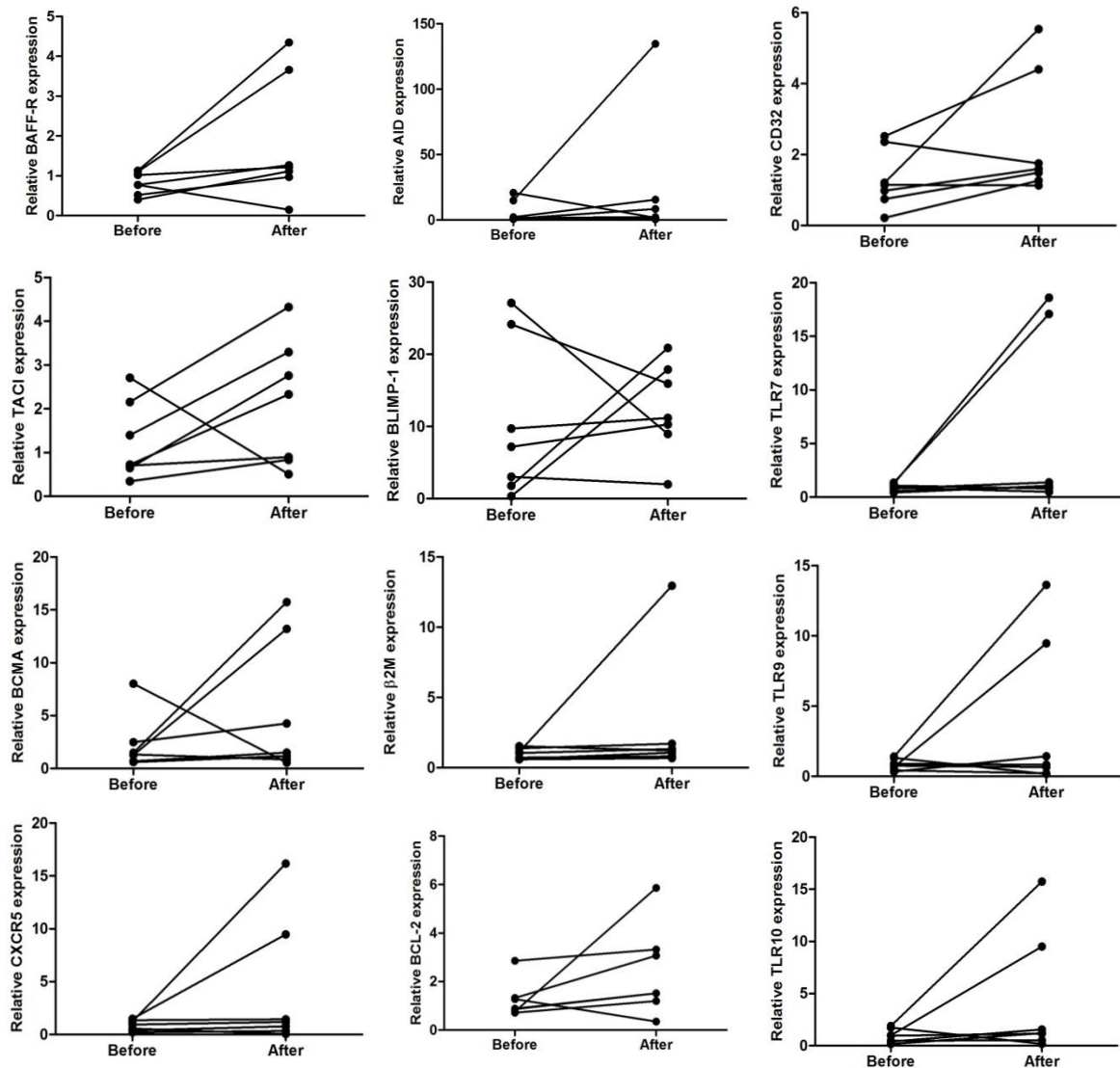


Fig. 10 - B cell gene expression is not affected by tocilizumab in established RA when compared to baseline. B cell gene expression levels of BAFF-R, TACI, BCMA, CXCR5, AID, BLIMP-1, B2M, BCL-2, CD32, TLR7, TLR9 and TLR10 were analyzed in established RA patients after 6 months of treatment with TCZ (n=7) and compared to baseline levels. No significant differences were observed in all B cell gene expression levels before and after treatment with TCZ.

3.4. Correlation analysis between B cell gene expression and clinical data

A correlation analysis was also performed between all the B cell gene expression levels of all groups included in this study with the clinical parameters previously indicated in Table 3. Nevertheless, no correlation was found between B cell gene expression with age, CRP, ESR, VAS, DAS28, swollen joints and tender joints in all patients' groups (data not shown). Furthermore, no correlation could be detected between B cell gene expression levels and age in the control group (data not shown).

6. DISCUSSION

In the present study, a group of genes directly related with B cell activation and survival (BAFF-R, TACI, BCMA), class-switching and somatic hypermutation (AID), plasma cell differentiation (BLIMP-1), chemotaxis (CXCR5), apoptosis (BCL-2), inhibition (CD32), inflammation (B2M) and B cell activation through TLRs (TLR7, TLR9, TLR10) was analyzed on peripheral blood isolated B cells from untreated early RA patients (ERA) (≤ 1 year of disease duration) and compared to established RA patients treated with MTX, MTX pre-biologic therapy, TNF inhibitors and TCZ for a better understanding about the effect of treatment options.

Our data has shown that B cell gene expression levels were surprisingly similar in ERA patients and healthy controls and, therefore no significant changes in B cell gene expression were detected during the first year of RA development. Previous studies by our group have found differences in B cell gene expression levels in patients with very early RA – VERA (< 6 weeks of disease duration) and ERA patients when compared to healthy controls, namely increased BAFF and TACI gene expressions¹⁰⁵. Nevertheless, this study was performed on PBMC and not on isolated B cells like the present work. Thus, we cannot exclude the possibility that different B cell proportions present in PBMC and purified B cell samples might affect the results obtained. Moreover, the number of patients included and patient variability can also influence the results observed.

Contrarily to ERA patients, significant alterations in B cell gene expression could be observed in established RA patients in comparison with controls, depending on the treatment. Indeed, disturbances in the gene expression levels were found in BAFF receptors (BAFF-R), TLRs (TLR7, TLR9, TLR10), inhibitory markers (CD32) and in genes associated with either active inflammation (B2M) or apoptosis (BCL-2) signaling pathways.

B cells can be activated in the context of T-dependent responses, which largely takes place in the germinal center and are associated with Ag stimulation through the BCR in the context of T cell–derived co-stimulatory signals, such as IL-21 and CD40L¹⁰⁶. Alternatively, B cells can be activated by T-independent antigens: for example, by signals delivered through TLRs and chronic activation of self-reactive B cells may be initiated through several pathways¹⁰⁷. In this regard, BAFF may substitute T cells in B cell activation¹⁰⁸. The receptors for this cytokine include BAFF-R, TACI and BCMA, all of which belong to the TNF family. BAFF-R is uniformly expressed in the majority of B cell lineage¹⁰⁹ and it has been associated with autoimmune diseases such as systemic lupus erythematosus (SLE)¹¹⁰ and

lymphoproliferative disorders ^{111–113}. Of note, an overexpression of BAFF-R has also been found in RA synovial tissue and circulating PBMC ¹⁰⁵. TACI is mainly expressed on memory B cells, plasma cells as well as on a subpopulation of activated CD27^{neg} B cells ^{109,114}; it also plays a role in class-switch recombination ¹¹⁵. BCMA expression is induced as a result of memory B cell differentiation into Ig-secreting plasma cells and its expression also occurs in fibroblast-like synoviocytes through the stimulation of APRIL (a proliferation-inducing ligand) ¹¹⁶ a cytokine that has affinity to BCMA and TACI receptors and is also implicated in B cell development ¹¹⁷.

From all BAFF receptors, BAFF-R was the only significantly increased in established RA patients treated with MTX and TNF inhibitors as compared to controls. Previous works have found increased BAFF-R gene expression levels in established RA treated with MTX or after anti-TNF therapy ¹⁰⁵, which further supports our results. Indeed, a study in type II collagen (CII)-induced arthritis (CIA) mice found that treatment with etanercept (a TNF inhibitor) was associated with an increase in BAFF-R expression and a negative correlation was found between the levels of BAFF-R and the percentage of memory B cells ¹¹⁸. Since established RA patients treated with TCZ had similar BAFF-R gene expression levels in comparison with controls, it seems that TCZ restores BAFF-R to normal gene expression levels. Of note, TCZ treatment was the most effective therapy in reducing disease activity (DAS28 score), which might justify the results obtained. The normal TACI gene expression observed in all patients' groups, irrespective of the treatment, might reflect B cell signaling mechanisms through other receptors. Furthermore, the absence of significant differences in BCMA gene expression are probably related to the low number of plasma cells in circulation, which are the main B cell subpopulation that express this cell marker.

Disturbances in gene expression levels of TLRs (TLR7, TLR9 and TLR10) were also detected in established RA patients. TLRs have been known to play central roles in infection and in driving inflammation in RA ^{107,119,120}. Furthermore, TLR signals *per se* are co-stimulatory and critically regulate T-independent autoreactive B cell activation and isotype-switch of Ig genes outside germinal centres ¹²¹. From the 10 human TLR subtypes, TLR1, 2, 4, 5 and 6 have extracellular expression to detect pathogenic elements in the surface, whereas TLR3 and TLR7-9 are found primarily in the intracellular endosome and detect internalized ligands. TLR7 binds to viral nucleic acids, whereas TLR9 recognizes unmethylated C-phosphate-G (CpG) bacterial DNA (reviewed in ¹²²). TLR10 function is still unknown but it has been speculated that it might cooperate with TLR2 in detecting lipoproteins ^{123,124}. TLR7 and TLR9 are mainly expressed in plasmacytoid dendritic cells and B cells, whereas TLR10 is highly expressed in B cells ¹²⁵. Previous studies have found that TLR7 and TLR9 mRNA

levels are up-regulated in PMBC of primary Sjögren's syndrome patients ¹²⁶. Of note, TLR7 has increased expression in RA synovium ¹²⁷. Moreover, TLR9 gene polymorphisms have been indicated as a possible susceptibility factor in RA ¹²⁸ and it has been suggested that TLR9 participates in the activation and proliferation of bone marrow B cells from RA patients ¹²⁹. Infection has long been speculated to be an underlying factor in RA pathogenesis, although it is still a controversial subject. The presence of peptidoglycan, bacterial and viral DNA has been shown in RA joints in previous studies, implicating TLRs' activation as a potential driver of the disease ¹³⁰. It is possible that B cell activation through BCR signalling pathway is inhibited with MTX and TNF inhibitors treatments and, consequently, B cells would readjust the expression levels of other activating receptors such as BAFF receptors or TLRs, which could justify the results obtained. Nevertheless, studies with a higher number of patients and also at the protein level would ideally clarify these results.

CD32 gene expression was also significantly increased in established RA patients after treatment with TNF inhibitors in comparison not only with controls, but also with ERA patients. Moreover, established RA patients treated with MTX pre-bio also had significantly increased CD32 gene expression levels when compared to ERA, although no significant differences were detected in comparison with controls. CD32 (FcγRII) is a transmembrane glycoprotein that mediates several functions including phagocytosis, cytotoxicity, immunomodulation and platelet aggregation ^{131,132}. CD32 is encoded by three genes (A, B, C) and different isoforms are generated via alternative mRNA splicing. Only B cells and plasma cells express the isoform IIb (FcγRIIb) ¹³³, which has been associated with SLE and RA. Indeed, genetic polymorphisms in FcγRIIb have been associated with SLE severity ^{134,135} and also with increased radiologic joint damage in RA ^{136,137}. It has been demonstrated that CD32 (FcγRIIb) triggering on B cells downregulates antibody production and may, in some circumstances, promote apoptosis ¹³¹. Furthermore, studies in mice models of arthritis have shown that FcγRII reduces joint inflammation by promoting endocytosis and clearance of immune complexes from the joint ¹³⁷. Therefore, it is possible that treatment of established RA patients with TNF inhibitors, but not MTX or TCZ, induces an increase in CD32 (FcγRIIb) expression that can inhibit B cell activation and disease progression.

β2-microglobulin (B2M) is required for the expression of cell surface molecules, including classical major histocompatibility complex (MHC) class I, CD1, Qa-1, and FcRn (neonatal Fc receptor), and for the development of CD8⁺, NKT, and CD3⁺ CD4⁻ CD8⁻ T cell subsets ^{138,139}, all of which may potentially impact the development of humoral autoimmunity. In this study, all groups of established RA displayed high levels of B2M gene expression, irrespective of treatment. This supports the existence of an inflammatory process associated with B cell

activation in RA patients, which is not abrogated by treatment with immunosuppressive DMARDs, either synthetic or biologic.

BCL-2 gene, which codes for an important anti-apoptotic protein ^{140–142}, had increased expression levels in all established RA groups, irrespective of treatment not only in comparison with controls, but also with ERA patients. BCL-2 transgenic expression in the B lineage results in B cell hyperplasia without an induction of cell cycle progression *in vivo*, and enhanced survival of peripheral B cells *in vitro* ^{143–145}. An increased expression of BCL-2 has been previously demonstrated in SLE patients and studies with transgenic animals indicate that dysregulation of BCL-2 may lead to autoimmune disease resembling human SLE ¹⁴⁵. RA is associated with an accumulation of inflammatory cells in the joints and their subsequent activation and proliferation, suggesting that pro-survival factors such as BCL-2 keep the peripheral B cell lifespan, thus contributing to disease persistence and progression. This effect does not seem to be abrogated by treatment options.

In addition, no significant differences were found in relative CXCR5, AID and BLIMP-1 gene expression in B cells. CXCR5 is a chemokine receptor expressed in recirculating B cells, in subsets of CD4⁺ and CD8⁺ T cells and monocytes ¹⁴⁶. Together with its ligand CXCL13 ¹⁴⁷, mediates migration of B cells into the follicles of the splenic white pulp, thus permitting antigen deposition on follicular dendritic cells ^{148,149}. It has been demonstrated that RA patients display upregulated expression of CXCR5 to a greater extent than other chemokine receptors, and CXCR5-null mice subjected to antigen-induced arthritis display reduced joint destruction ^{150,151}. Although we did not find significant differences in CXCR5 gene expression in all groups, we cannot exclude the hypothesis that the expression of this chemokine receptor might have been affected at the protein level. AID, a member of the cytidine deaminase family, is involved in somatic hypermutation and class-switch recombination of immunoglobulin genes, which take place in the GC. Indeed, the molecular mechanism of cytosine deamination induces immunoglobulin affinity maturation and antibody class switching ¹⁵². It has been demonstrated that AID transcripts are increased in rheumatoid synovitis ¹⁵³. Thus, although there were no significant differences in AID gene expression levels in circulating B cells, it is possible that locally in the joints higher levels of AID gene expression can be found. BLIMP-1 is a transcription factor and regulates the terminal differentiation of many types of cells ¹⁵⁴, including the differentiation of B cells into plasma cells ¹⁵⁵. Most plasma cells formed after a T cell-dependent GC reaction secrete antibodies with higher affinity and switched isotypes and infiltrate the synovial membrane ^{156,157}. The reduced number of circulating plasma cells (usually less than 2-3 %) ⁹⁴ might justify the

results observed. Similarly to AID, BLIMP-1 gene expression levels are probably increased in rheumatoid synovium, which is highly infiltrated by antibody-secreting cells.

Overall, disturbances in B cell gene expression levels directly related to B cell activation (BAFF-R, TLR7, TLR9 and TLR10) and/ or inhibition (CD32), inflammation (B2M) and apoptosis (BCL-2) are present in later stages of RA development. At earlier stages of RA, patient variability and disease activity might also affect B cell gene expression levels. Furthermore, the effect of treatment is more evident when results are compared to healthy individuals and not with baseline values. Nevertheless, larger cohorts of patients and/ or analysis at the protein level would be required to allow more robust conclusions that enlighten the pathways involved in RA.

7. LIMITATIONS AND FUTURE PERSPECTIVES

Despite the efforts, it is still important to keep introducing new treatments for RA to overcome problems associated with the various factors triggering the disease. Treatments for RA have been advancing rapidly and new ones are now on clinical trials ⁷⁸. The drugs discussed in this study appear to be relatively safe and effective in short-to-intermediate term and although the effects seem promising, searching for new biomarkers could be very useful to achieve a better treatment efficacy. Moreover, it would also be important to study the effects of treatment options individually in particular all TNF inhibitors available (infliximab, adalimumab, certolizumab, golimumab and etanercept), since it is possible that different drugs can have different effects. Of note, it would also be interesting to study other therapeutic approaches such as the B cell depleting drug rituximab and/ or abatacept, a fusion protein composed of the Fc region of the immunoglobulin IgG1 fused to the extracellular domain of CTLA-4 that inhibits T cell activation.

In addition, it would also be important to study individual B cell subpopulations not only at the gene level, but also at the protein level to better understand the relevance of all B cell subsets in RA development and progression. Nevertheless, the collection of larger patient cohorts, which can be a challenging issue in biomedical investigation, would be necessary in order to have a more robust and conclusive analysis.

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